



## Adipose-derived stem cells for treatment of chronic wounds

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# **ADIPOSE-DERIVED STEM CELLS FOR TREATMENT OF CHRONIC WOUNDS**

**BY  
SIMONE ELKJÆR RIIS**

DISSERTATION SUBMITTED 2016



**AALBORG UNIVERSITY**  
DENMARK



# **ADIPOSE-DERIVED STEM CELLS FOR TREATMENT OF CHRONIC WOUNDS**

**PHD DISSERTATION**

by

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**AALBORG UNIVERSITY**  
DENMARK

Laboratory for Stem Cell Research

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# CV



## Résumé

I am born on 26th of June 1987 in a small village in eastern Jutland, Ryomgaard. Here I lived in safe surroundings with my parents and younger brother for 20 years. Now the time had come to move on and I moved to Aalborg to take my dream education. I kind of got stuck in the city, found the love of my life and now I am finishing my Ph.D. at Aalborg University.

## Education and Qualifications

- 2012-2015 Ph.D. student at Clinical Science and Biomedicine, AAU
- 2012- Teaching at Medicine and Medicine with Industrial Specialization, AAU
- 2012 Project Management, AAU
- 2010-2012 Master in Medicine with Industrial Specialization – Biomedicine, AAU
- 2007-2010 Bachelor in Medicine with Industrial Specialization, AAU

## Employment

- 2015- Research assistant, Department of Health Science and Technology, AAU
- 2012-2015 PhD fellow, Department of Health Science and Technology, AAU

## Teaching

Semester coordinator on 4. semester (2016), module coordinator on 1. and 2.semester (2015-), case facilitator on 1., 2. and 4. semester (2012-), project supervision on 4. Semester (2014, 2016), lectures on 1., 2., 4., and 8. Semester (2013-), workshops on 1. and 2. Semester (2012-).

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## Publications

Critical steps in the isolation and expansion of adipose-derived stem cells for translational therapy. / Riis, S.; Zachar, V.; Boucher, S.; Vemuri, M.; Pennisi, C. P.; Fink, T. *Expert Reviews in Molecular Medicine*, Vol. 17, e11, 2015.

Comparative analysis of media and supplements on initiation and expansion of adipose-derived stem cells. / Riis, S.; Nielsen, F. M.; Pennisi, C. P.; Zachar, V.; Fink, T. *Stem Cells Translational Medicine*. In press.

Activation of protease-activated receptor 2 induces VEGF independently of HIF-1. / Rasmussen, J.; Riis, S.; Frøbert, O.; Yang, S.; Kastrup, J.; Zachar, V.; Simonsen, U.; Fink, T. *P L o S One*, Vol. 7, Nr. 9, 25.09.2012, s. Article No. e46087.

Effect of unaccustomed eccentric exercise on proprioception of the knee in weight and non-weight bearing tasks. / Vila-Cha, C.; Riis, S.; Lund, D.; Møller, A.; Farina, D.; Falla, D. *Journal of Electromyography & Kinesiology*, Vol. 21, No. 1, 2011, p. 141-147.

Hypoxia augments the wound healing effects of adipose-derived stem cells in primary human keratinocyte scratch assay. / Riis, S.; Newman, R.; Andersen, J.; Kuninger, D.; Boucher, S.; Vemuri, M., Pennisi, C. P., Zachar, V., and Fink, T. *Submitted*.

Proteome analysis of adipose-derived stem cells cultured under clinically relevant conditions in a wound healing perspective. / Riis, S.; Stensballe, A.; Emmersen, J.; Pennisi, C. P.; Birkelund, S.; Zachar, V.; Fink, T. *Submitted*.

Comparative analysis of subpopulations of adipose-derived stem cells. Nielsen, F. M.; Riis, S.; Andersen, J.; Fink, T.; Zachar, V. *Manuscript in preparation*.

### **Conference abstracts**

Investigation of ASC-mediated wound healing in in vitro skin injury models. / Riis, S.; Newman, R.; Kuninger, D.; Boucher, S.; Vermuri, M.; Zachar, V.; Fink, T.. *Cytotherapy*, Vol. 16, Nr. 4, Suppl., Abstract No. 326, 2014, s. S93.

In human adipose stem cells trypsin treatment upregulates expression and secretion of VEGF in a manner independent of hypoxia inducible factor 1. Fink, T.; Rasmussen, J.; Riis, S.; Lundsted, D.; Larsen, B.; Frøbert, O.; Kastrup, J.; Simonsen, U.; Zachar, V. *International Federation for Adipose Therapeutics and Science*. Conference 2011.

# PREFACE

This thesis: *Adipose-derived stem cells for treatment of chronic wounds* has been submitted to the Faculty of Medicine, Aalborg University, Denmark. The experimental work in this thesis has been carried out at the Laboratory for Stem Cell Research, the Biomedicine Group, Department of Health Science and Technology, Aalborg University, Denmark, at the Laboratory for Medical Mass Spectrometry, the Biomedicine Group, Department of Health Science and Technology, Aalborg University, Denmark and in the research facilities at Life Technologies, Frederick, MD, USA from September 2012 to February 2016.

In the autumn 2013 I spend 6 weeks in the research facilities of the Primary and Stem Cell Systems group of Life Technologies, Thermo Fischer Scientific Inc., Frederick, MD, USA. Here I worked with human primary keratinocytes in the attempt to establish a 3D wound healing model.

During my PhD study I have been teaching, coordinating teaching, supervising and examining students attached to the two specializations Medicine with Industrial Specialization and Medicine, and I have completed PhD courses corresponding to 30 ECTS points. Together these activities correspond to more than a full year of my PhD study.

The thesis is based on four experimental studies and a review. One is in press, two has been submitted and one is in preparation for submission. Additionally, the review has been published. The thesis is composed of a general introduction encompassing the topics being explored in the manuscripts, the aim and objectives of the thesis, selected methods are presented, the results are presented as a summary of the four manuscripts, a general discussion and finally conclusions and perspectives. The original research articles and the review are attached as appendix.



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Director for Cell Biology Mohan Vemuri, Senior Staff Scientist and Primary Cells R&D Group Leader David Kuninger, Ph.D., Staff Scientist Shayne Boucher, Ph.D. and Senior Staff Scientist Rhonda Newman, Ph.D., and all the others at the Primary and Stem Cell Systems group and the Frederick site of Life Technologies, Thermo Fischer Scientific Inc., are acknowledged for making my stay a possibility and such a pleasant experience. Their open arms and helpful minds were really remarkable.

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Without all of You, this thesis would never have been possible ♥.

# ABSTRACT

The aim of this thesis is to aid the translation of adipose-derived stem cells (ASCs) into clinical use in wound healing.

To achieve this, two central objectives were determined: 1) to identify the mode of action of ASCs in wound healing and how this is affected by hypoxic culture, and 2) to identify how *in vitro* procedures of isolation and expansion affect ASC properties.

To achieve the first objective, ASCs were cultured in 20% or 1% O<sub>2</sub> and their pro-epithelialization properties analyzed. As a measure for this, a primary keratinocyte based scratch assay was implemented. Additionally, the influence of hypoxic culture on the secretome and proteome of ASCs was analyzed using LC-MS/MS. To achieve the second objective, ASCs were isolated and cultured in a variety of basal media and supplements, and the effect of these was analyzed in terms of cell attachment, proliferation, CFU, and surface marker profile. Additionally, the subpopulations of the heterogeneous ASCs were analyzed in terms of differentiation capacity, proportion of CFUs, and ability to promote endothelial migration.

This showed that conditioned media from ASCs promoted the *in vitro* wound healing of keratinocytes, and this was also increased when using conditioned media from hypoxically preconditioned cells. When investigating the effect of hypoxic preconditioning on the ASC proteome, it was found that ECM relevant proteins were upregulated. When investigating the effect of *in vitro* culture on the ASCs, it was found that choice of media affects the properties of the ASCs. Furthermore, the different subpopulations within ASCs do have different pro-angiogenic characteristics. However, by passaging the subpopulations, the surface marker profile returns back to that of the original mixed population.

In conclusion, ASCs were shown to promote wound healing through the promotion of re-epithelialization and ECM remodeling, and hypoxic preconditioning of ASCs showed to enhance both. The establishment of the ASCs showed to be affected by the choice of basal medium and supplement, and the distinct subpopulations of the ASCs showed to have differences in the functional properties. Hopefully, these findings can aid the translation of ASCs into clinical use in wound healing.





# DANSK RESUMÉ

## (Abstract in Danish)

Formålet med denne afhandling er at fremme den kliniske brug af adipøse stamceller til heling af kroniske sår.

For at opfylde dette blev to målsætninger opsat: 1) at identificere virkemåden af adipøse stamceller under sårheling og undersøge hvordan denne påvirkes af hypoksi og 2) at identificere, hvordan isolerings- og ekspanderingsprocedurer påvirker stamcellernes egenskaber.

For at opnå den første målsætning blev adipøse stamceller først dyrket under enten 20% eller 1% ilt. Herefter blev deres evne til at støtte re-epithelialisering analyseret gennem brugen af et forsøg baseret på humane primære keratinocytters evne til at lukke en rift. Herudover blev effekten af hypoksi på stamcellernes proteiner analyseret ved hjælp af massespektrometri.

For at opnå den anden målsætning blev adipøse stamceller isoleret og dyrket i en række kombinationer af forskellige basale vækstmedier og mediesupplementer. Disse medie kombinationer blev undersøgt for deres indflydelse på cellernes adhesion til plastik, proliferation, evne til at danne kolonier samt sammensætningen af overflademarkører. Herudover blev forskellige subpopulationer af stamcellerne undersøgt, og disses evne til at differentiere, danne kolonier og støtte endothelceller bestemt.

Vores undersøgelser viste, at adipøse stamceller påvirker sårheling positivt ved at øge re-epithelialiseringen og at hypoksi fremmer stamcellernes effekt. Ved at kigge nærmere på stamcellernes proteiner blev det fundet, at hypoksi øger tilstedeværelsen af proteiner involveret i moduleringen af den ekstracellulære matrix. Ydermere fandt vi, at dyrkning af adipøse stamceller påvirker deres egenskaber afhængigt af det brugte vækstmedie. De forskellige subpopulationer viste sig at have forskellige pro-angiogene egenskaber, dog returnerede sammensætningen af overflademarkørerne for de forskellige subpopulationer til den oprindelige blandede sammensætning når disse var ekspanderet.

Det kan derfor konkluderes, at adipøse stamceller fremmer sårheling gennem en øget re-epithelialisering, som kan øges yderligere ved brug af hypoksi. Hypoksi påvirker stamcellernes proteiner på en måde, der medfører en øget sårhelende effekt. Isoleringen og ekspanderingen af adipøse stamceller viste sig at være påvirket af valg af vækstmedie, og stamcellernes subpopulationer havde forskellige funktionelle egenskaber. Disse fund kan forhåbentligt støtte op om den kliniske brug af adipøse stamceller til heling af kroniske sår og fremme deres anvendelse.



# LIST OF MANUSCRIPTS

## Study 1:

Hypoxia augments the wound healing effects of adipose-derived stem cells in primary human keratinocyte scratch assay. / Riis, Simone; Newman, Rhonda; Andersen, Jens; Kuning, David; Boucher, Shayne, Vemuri, Mohan, Pennisi, Cristian Pablo, Zachar, Vladimir, and Fink, Trine. *Submitted*.

## Study 2:

Proteome analysis of adipose-derived stem cells cultured under clinically relevant conditions in a wound healing perspective. / Riis, Simone Elkjær; Stensballe, Allan; Emmersen, Jeppe; Pennisi, Cristian Pablo; Birkelund, Svend; Zachar, Vladimir; Fink, Trine. *Submitted*.

## Study 3:

Comparative analysis of media and supplements on initiation and expansion of adipose-derived stem cells. / Riis, Simone Elkjær; Nielsen, Frederik Mølgaard; Pennisi, Cristian Pablo; Zachar, Vladimir; Fink, Trine. *Stem Cells Translational Medicine. In press*.

## Study 4:

Comparative analysis of subpopulations of adipose-derived stem cells. Nielsen, Frederik Mølgaard; Riis, Simone Elkjær; Andersen, Jens; Fink, Trine; Zachar, Vladimir. *Manuscript in preparation*.

## Review:

Critical steps in the isolation and expansion of adipose-derived stem cells for translational therapy. / Riis, Simone Elkjær; Zachar, Vladimir; Boucher, Shayne; Vemuri, Mohan; Pennisi, Cristian Pablo; Fink, Trine. *Expert Reviews in Molecular Medicine*, Vol. 17, e11, 2015.



# LIST OF ABBREVIATIONS

ASC	Adipose-derived stem cell
bFGF	Basic fibroblast growth factor
BM-MSCs	Bone marrow mesenchymal stem cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FCS	Fetal calf serum
GMP	Good manufacturing practice
HGF	Hepatocyte growth factor
HIF-1	Hypoxia-inducible factor-1
hPL	Human platelet lysate
IFATS	International Federation for Adipose Therapeutics and Science
IGF	Insulin-like growth factor
IL	Interleukin
ISCT	International Society for Cellular Therapy
KGF	Keratinocyte growth factor
MMP	Matrix metalloproteinases
MS	Mass spectrometry
MSC	Mesenchymal stem cells
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PDGF	Platelet-derived growth factor
PTM	Post-translational modification
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
SVF	Stromal vascular fraction
TGF- $\beta$	Transforming growth factor- $\beta$
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
VEGF	Vascular endothelial growth factor



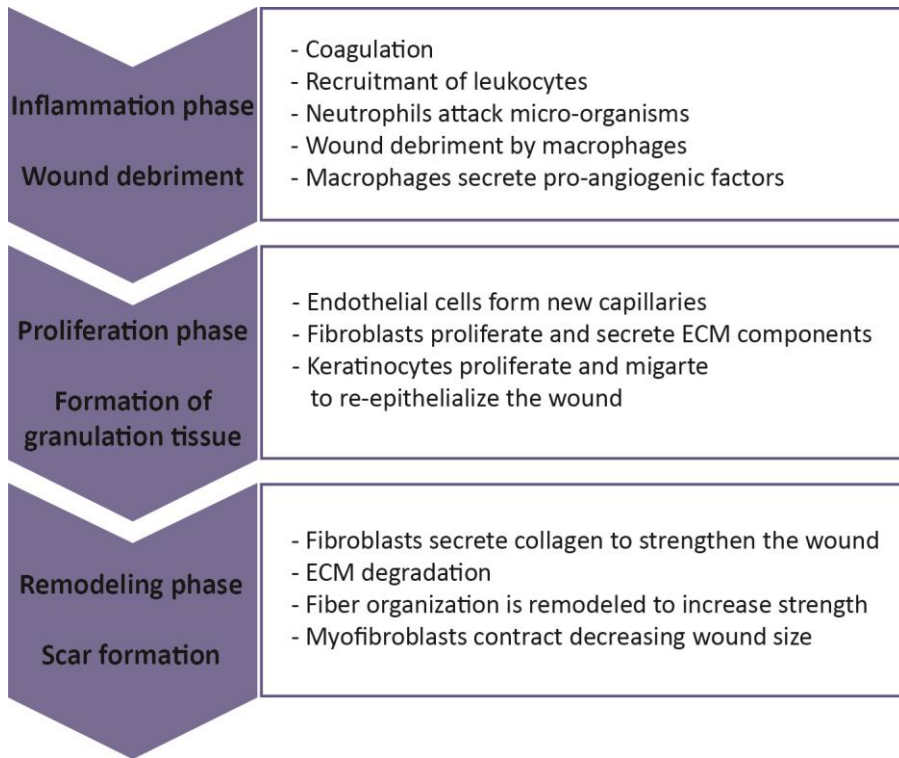
# CHAPTER 1. INTRODUCTION

## 1.1. CHRONIC WOUNDS

Chronic wounds are defined as non-healing wounds or ulcers that persist for more than 6 weeks (Markova and Mostow, 2012). Commonly they develop after minor injuries accompanied by advanced age and medical comorbidities such as diabetes (Demidova-Rice et al., 2012).

Chronic wounds affect millions of people around the world (Demidova-Rice et al., 2012) and as the general population advances in age and the prevalence of lifestyle diseases as obesity, diabetes and venous insufficiency increases, chronic wounds are becoming an increasing health burden especially in the industrialized countries (Zielins et al., 2014). During a lifetime, 1 - 2% of the population in the industrialized countries will experience a chronic wound (Sen et al., 2009). To the patient, having a chronic wound diminishes quality of life due to significant functional impairment, psychosocial morbidity and an increased risk of limb amputation (Zielins et al., 2014). The economic costs of wound care are estimated to constitute around 2 - 4% of the total health-care budget within the EU (Hjort and Gottrup, 2010), and in the US alone, wound care is estimated to be a >25 billion dollar business (Brem et al., 2007). However, on top of this should be added loss of productivity for both patients and relatives, which also constitute a significant sum.

In normal acute wounds, healing begins directly after an injury occur in three sequential, highly integrated and spatially overlapping phases; inflammation, proliferation and remodeling (Baum and Arpey, 2005)(Figure 1). During the inflammation phase, a fibrin cloth is formed, neutrophils are recruited, mast cells mature, other immune cells, fibroblasts and keratinocytes are activated and arriving monocytes differentiate into macrophages (Ng, 2010). The purpose of this phase is to stop the bleeding, clean out infectious agents, and initiate tissue re-generation. During the proliferation phase, fibroblasts are attracted into the wound synthesizing extracellular matrix (ECM) components and myofibroblasts contract, decreasing the wound size. Additionally, endothelial cells migrate into the wound beginning to form new blood vessels by the process of angiogenesis (Bao et al., 2009) and keratinocytes, residing in the wound edges and hair follicles, proliferate, begin to migrate into the wound and finally differentiate to re-epithelialize the wound (Baum and Arpey, 2005). During the remodeling phase, the cellular content of the scar tissue is decreased due to migration and apoptosis of the residing cells and the type III:type I collagen ratio decreases changing the composition and the properties of the ECM (Baum and Arpey, 2005). The remodeling phase begins in average three weeks after injury and can last for years depending on the severity of the wound. If any of these events is disturbed, the healing will pause, and the wound can become chronic (Hassan et al., 2014).



*Figure 1. Phases of wound healing and the cellular events. ECM: extracellular matrix. Modified from (Falanga, 2005).*

Chronic wounds encompass several categories of non-healing wounds with a variety of etiologies, but most commonly they are categorized as vascular, diabetic or pressure ulcers. Vascular ulcers occur secondarily to decreased blood-dermis oxygen exchange either caused by venous valvular ineffectiveness or arterial insufficiency (Demidova-Rice et al., 2012). Diabetic ulcers occur as a result of the combination of neuropathy, vascular impairment, muscle metabolic deficiencies, and microvascular pathologies caused by hyperglycemia (Falanga, 2005; Leung, 2007). Pressure ulcers develop due to prolonged unrelieved pressure and shearing forces on the tissue, leading to decreased oxygen tension, ischemia reperfusion injury and necrosis (Demidova-Rice et al., 2012). A common feature of the different types of chronic wounds is a lack of cellular response to the environmental cues (Demidova-Rice et al., 2012). This includes a lack of conversion from the pro-inflammatory M1 macrophages to the anti-inflammatory M2 macrophages (Manning et al., 2015) and overactive mast cells (Yun et al., 2012) both resulting in persistent inflammation. Additionally, a general tendency of impaired cell proliferation and migration is observed in terms of both endothelial cells leading to insufficient angiogenesis and keratinocytes disturbing the process of re-epithelialization (Demidova-Rice et al.,



2012; Raja et al., 2007). Furthermore, the microenvironment of the chronic wounds is dominated by an increased presence of matrix metalloproteinases (MMPs) which degrade ECM and other proteases that degrade extracellular growth factors (Saarialho-Kere, 1998) as hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), transforming growth factor- $\beta$  (TGF- $\beta$ ), and vascular endothelial growth factor (VEGF) which all are important wound healing factors (Greaves et al., 2013; Hassan et al., 2014).

The goal of the treatment strategies for chronic wounds is to return the wound to the progression of normal wound healing. The conventional treatments are based on an element of wound bed preparation using tissue debridement, a treatment of infection using topically administered antibiotics, maintaining a properly moist environment by applying ointment or using negative pressure therapy, and in severe cases also surgical repair (Frykberg and Banks, 2015; Werdin et al., 2009). Nonetheless, a large percentage of patients do not respond to these treatments or their wounds reoccur. To overcome this and to obtain a satisfactory treatment outcome, it is believed that the residing cells need to be stimulated (You and Han, 2014). Many approaches towards this have been attempted as supplement to the conventional wound management, though, without evidence of the effects of these (Frykberg and Banks, 2015; Sundhedsstyrelsen, 2011; Werdin et al., 2009; You and Han, 2014).

## 1.2. ADIPOSE-DERIVED STEM CELLS

A new and interesting alternative therapeutic strategy for repairing damaged tissue is regenerative medicine, which is based on the process of regenerating human cells, tissues or organs to restore normal function by stimulating the body's own repair mechanism to heal tissue or organ defects. Within regenerative medicine stem cells have shown great promise. Stem cells are believed to be part of the internal repair system of the body, where they replace cells that are lost due to normal turn-over or pathological conditions. They are unspecialized cells capable of dividing asymmetrical, thereby continuously renewing themselves and giving rise to specialized cell types (Ding et al., 2011; Hassan et al., 2014; Zuk, 2013).

A variety of stem cells can be found during the life time of the human body. In the stages of development, embryonic stem cells from the blastocyst give rise to all cell types of the body and later somatic stem cells maintain the integrity of the organism and can be found in nearly all tissues. The use of embryonic stem cells in research is decreasing due to high risk of teratoma development and the moral and ethical concerns around their origin. Somatic stem cells are multipotent, and are able to differentiate into a limited number of cell types; often those originating from the same germ layer as the stem cell itself originate. A type of somatic stem cells is mesenchymal stem cells (MSCs), which are derived from the mesodermal embryonic tissue. The MSCs can be found in connective tissues and the most used MSCs have been bone marrow mesenchymal stem cells (BM-MSCs). However, due

to the clinical limitations of bone marrow biopsies alternative sources have been sought. This led to the discovery of related MSCs in adipose tissue, termed adipose-derived stem cells (ASCs), which in large have shown same biological capabilities as the BM-MSCs (Zuk et al., 2002). The advantages of ASCs over BM-MSCs and other stem cell types are that ASCs are relatively easy to obtain from liposuctions performed in local anesthesia, they can be obtained in a large numbers, they are capable of maintaining their phenotype and plasticity after long term *in vitro* culture and they comprise a low immunogenicity (Cherubino et al., 2011; Zuk, 2013). Based on this ASCs have generated great interest and is by some perceived as the most preferred cell type for regeneration and wound repair.

ASCs are found in adipose tissue amongst adipocytes, endothelial cells, fibroblasts and immune cells (Ye and Gimble, 2011). They are located in the perivascular space, where they seem to function in the repair of injured tissue and in interaction with other cells according to the stimuli they receive (Lee et al., 2009). ASCs are a heterogeneous population of cells with an overall fibroblastic morphology, large endoplasmatic reticulum and large nuclei (Gimble and Guilak, 2003). Originally, they were defined by the trilineage differentiation potential along with plastic adherence, and a narrow surface marker profile (Zuk et al., 2001). As more and more research have been carried out, other more clinically relevant parameters have been identified and today a broader panel of surface markers have been suggested and also the paracrine properties are of special interest (Bourin et al., 2013).

### 1.3. ASCS IN WOUND HEALING

In pre-clinical studies, ASCs have shown great promise as a treatment modality for healing of cutaneous wounds (Isakson et al., 2015; Zamora et al., 2013; Zografou et al., 2013).

The mechanism of action by which ASCs and other mesenchymal stem cells accelerate skin regeneration and wound healing is not fully understood. Initially, stem cells were assumed to home the injured tissue and repair defects by differentiating into the specific cells needed for tissue regeneration and therefore the initial clinical translation focused on the differentiation potential (Chung et al., 2009). However, the validity of this theory has been questioned due to a limited survival of engrafted stem cells and the limited differentiation potential of ASCs not encompassing neither dermal fibroblast nor keratinocyte lineage differentiation, which would be required for complete wound healing by differentiation (Song et al., 2010). Consequently, it has been hypothesized, that ASCs exert their wound healing properties through the stimulation and modulation of the residing tissue cells through the secretion of soluble factors (Rehman et al., 2004). Supporting this theory, ASCs have been found to secrete several growth factors and cytokines involved in wound healing including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), HGF, insulin-like growth factor (IGF), PDGF,

TGF- $\beta$ , and VEGF (Zhao et al., 2013). These observations have shifted the focus for clinical use of ASCs.

Different studies have tried to explain the wound healing properties of ASCs more in depth by examining their effect on single aspects in each of the different phases of wound healing. Relevant to the inflammation phase, ASCs have been shown to have immunomodulatory effects (Cui et al., 2007; Gonzalez-Rey et al., 2010; Kuo et al., 2011; Melief et al., 2013) which are mediated through secretion of both pro- and anti-inflammatory cytokines as IL-6, IL-8, and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and IL-10, HGF, and TGF- $\beta$  respectively (Lee et al., 2010; Melief et al., 2013; Strong et al., 2015; Zhao et al., 2013). Additionally, ASCs are capable of promoting the transition of macrophages from a pro-inflammatory M1 phenotype associated with chronic wounds to the anti-inflammatory M2 phenotype normally present in later stages of normal wound healing (Manning et al., 2015). The effect on the macrophages is probably mediated through the secretion of IL-4, IL-10 and IL-13 (Cho et al., 2014; Zhao et al., 2013). Moreover, they have been shown to decrease the activity of mast cells (Yun et al., 2012). Thus it is conceivable, that ASCs may promote the transition of the wound healing from the inflammation phase into later stages of the healing process.

During the proliferation phase, positive effects of ASCs have been found in terms of supporting the formation of granulation tissue. They have been shown to be pro-angiogenic, as conditioned medium from ASCs resulted in enhanced endothelial sprouting, migration rate, cell viability, and endothelial tube formation (Nakagami et al., 2005; Rasmussen et al., 2011; van der Meer et al., 2010). Additionally, ASCs have been shown to increase fibroblast migration and proliferation through the secretion of bFGF and EGF (Zhao et al., 2013). Finally, the re-epithelialization has been shown to be promoted by ASCs (Alexaki et al., 2012; Lee et al., 2012) possibly through the secretion of cytokines as EGF, bFGF, HGF, IGF-1, keratinocyte growth factor (KGF) and PDGF, which stimulates keratinocyte proliferation and migration (Barrandon and Green, 1987; Tsuboi et al., 1993, 1992).

Relevant to the remodeling phase, ASCs have been shown to have a positive effect on scarring, by decreasing size and color and increasing pliability, possible due to a timely controlled increase in the expression of MMPs (Yun et al., 2012). Additionally, ASCs have shown to decrease the ratio of type III:type I collagen by increasing the secretion of type I collagen into the wound ECM (Cho et al., 2010; Lee et al., 2009). This might be augmented by their secretion of IL-10, which has been shown to increase the expression of MMPs in fibroblasts preventing and reducing skin scarring through inhibiting excessive ECM deposition (Shi et al., 2013).

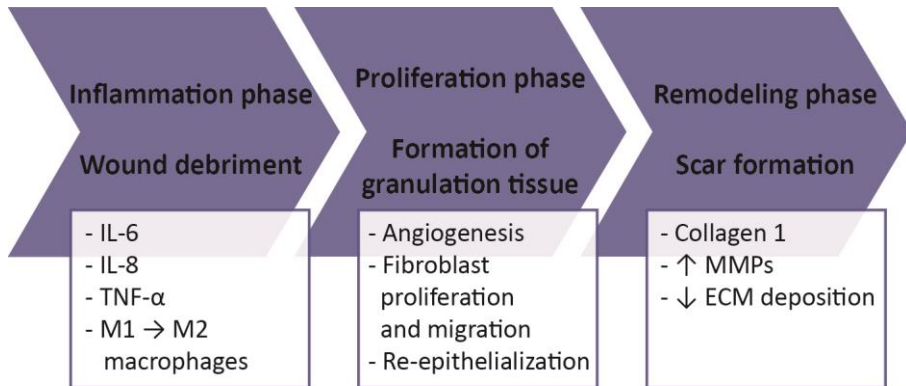


Figure 2. The effect of ASCs on the phases of wound healing. IL, interleukin; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; MMP, matrix metalloproteinase; ECM, extracellular matrix.

The indications of ASCs being responsive to their environment and that they exhibit a multi action pro-wound healing response supports the use of ASCs as a treatment modality for the healing of chronic wounds, especially with the mixed etiology of these.

## 1.4. CLINICAL USE

Today, ASCs are used in a row of early clinical trials where their safety and efficacy have been tested and shown great promise (Cho et al., 2013; De La Portilla et al., 2013; Garcia-Olmo et al., 2009; García-Olmo et al., 2005; Herreros et al., 2012; Lee et al., 2012, 2013). These trials investigate the use of ASCs as a treatment of ischemic heart disease, limb ischemia, and fistulas amongst others.

To translate the pre-clinical findings of ASCs promoting wound healing into clinical trials several practical aspects are to be considered and the influence of these on the ASCs are to be investigated. A full review of these aspects can be found in (Riis et al., 2015).

### Autologous vs. allogenic donors

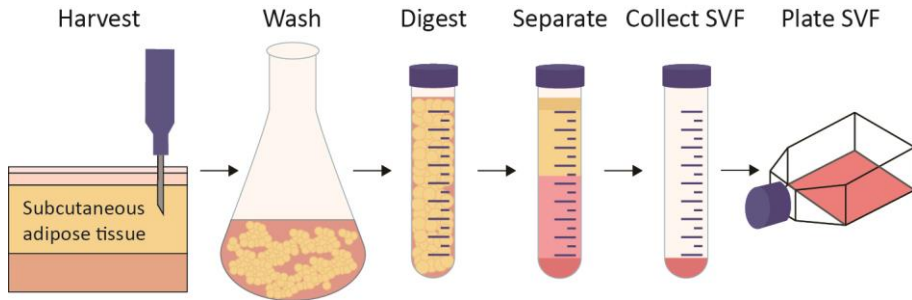
When choosing stating material for obtaining ASCs, a number of aspects have to be taken into consideration. One of these is the use of autologous vs. allogenic ASCs. Previously, concerns of using allogenic stem cells have been raised due to safety issues and the risk of immune rejection, and thereby lack of therapeutic effect. The majority of research has therefore been based on autologous ASCs by which the risk of immune rejection could be reduced (Ra et al., 2011). These studies have shown the use of autologous ASC to be safe and effective (Marino et al., 2013; Ra et al., 2011). However, to use autologous ASCs it is necessary for the patient to undergo the liposuction and the reinjection procedures at two different time points increasing

the time frame as well as the costs of the treatment. Additionally, patients with chronic wounds often suffer from comorbidities affecting the quality of the stem cells, reducing the regenerative properties of these (Cianfarani et al., 2013). Recently, the use of allogenic ASCs have been more accepted as studies have shown autologous and allogenic mesenchymal stem cells to be comparable in terms of immunomodulatory properties and their ability to evade or suppress the immune system (Cui et al., 2007; De Miguel et al., 2012; Gonzalez-Rey et al., 2010). Additionally, allogenic ASCs have shown clinical effects within the area of autoimmune diseases (Fang et al., 2007a, 2007b, 2007c; Voswinkel et al., 2013). Using allogenic ASCs also makes a large scale production possible, thereby reducing patient waiting time, running costs and enables the treatment of a large number of patients with the same product enabling comparison of treatment efficacy and decreasing variability herein. However, more research is needed using allogenic ASCs before these can be used as a standard option and one should keep in mind that inter-recipient variation still might complicate the direct comparison of the outcome due to the disease heterogeneity.

### **Isolation procedure**

Through the years, different approaches have been used for the collection of adipose tissue. These are including and combining manual or pump assisted liposuction, resection, narrow or wide cannulas, and different types of local anesthesia. Only the choice of anesthesia showed to have an effect where lidocaine was to prefer. Otherwise no significant effect of any of the parameters in the tissue collection process on the biological properties of ASCs has been found (Aguena et al., 2012; Buschmann et al., 2013; Fraser et al., 2007; Muscari et al., 2012; Schreml et al., 2009). Thereby, liposuction seems to be the most widely used as it is both relatively simple, less invasive and the risk of complications is relatively low compared to tissue resection.

After the adipose tissue has been collected, the stem cells are to be isolated. The principle for this was first described by Zuk et al. (Zuk et al., 2001), and during the years many modifications have been used by different laboratories. Basically, the adipose tissue is first washed to remove erythrocytes, digested with a protease to disrupt the ECM, centrifuged to separate and remove adipocytes and tissue remnants, lysed to remove remaining erythrocytes and lastly seeded on a plastic surface to select ASCs from the rest of the stromal vascular fraction (SVF) being erythrocytes, leukocytes, immature adipocytes, and endothelial cells. After a few passages the adherent non-proliferative cells are outgrown by the ASCs which then are defined as pure. However the ASC culture is nonetheless still a pretty heterogeneous population. No changes to the original isolation protocol giving a significant better cell yield have been suggested, but different laboratories have their standards and preferred practices. However, emphasized is the necessity of using reagents of a good quality and controlled procedures to meet the standards of good manufacturing practice (GMP).



*Figure 3. Overview of the isolation process. Adipose tissue is harvested by liposuction and washed in phosphate buffered saline to remove blood remnants. The resulting adipose tissue is enzymatically digested and separated by centrifugation into a layer of lipids from disrupted mature adipocytes, a layer of mature adipocytes a liquid layer and in the bottom the stromal vascular fraction. To collect the SVF all layers above are removed. The SVF can then be re-suspended in culture medium and seeded on a plastic surface to select ASCs from the SVF.*

### Expansion process

After isolation and selection of ASCs, these are often to be expanded *in vitro* to obtain a clinically relevant number of ASCs. For expansion of ASCs different aspects are to be considered as an optimal cell growth depends on factors as oxygen tension (Fink et al., 2008; L. Pilgaard et al., 2009; Prasad et al., 2014; Rasmussen et al., 2012, 2011), culture surface (Foldberg et al., 2012; Pennisi et al., 2013), and the culture media compositions (Lund et al., 2009; Riis et al., 2015; Yang et al., 2012). The culture media is often composed of a basal medium and some kind of supplement. A variety of choices and combinations have been tested in the hope of finding one that supports the stem cell characteristics, avoid senescence and allows for a high proliferative rate (Parker et al., 2007; L. Pilgaard et al., 2009). Besides supporting stem cell maintenance, the media must also comply with GMP-standards and be approved for clinical application. To meet all of these requirements, an increasing number of alternatives to the use of fetal calf serum (FCS), which were originally suggested and have primarily been used for expansion of ASCs (Zuk et al., 2001), are being developed and commercialized. These include supplements as human platelet lysate (Hemeda et al., 2014; Naaijken et al., 2012; Trojahn Kølke et al., 2013) and xeno- and serum-free medium solutions as MesenCult™-XF from STEMCELL Technologies (Al-Saqi et al., 2014), StemPro MSC SFM XenoFree from Life Technologies now Thermo Fisher (Chase et al., 2012; Lindroos et al., 2009; Patrikoski et al., 2013; Yang et al., 2012). However the very complex composition of FCS including essential nutrients and bioactive molecules is very difficult to reproduce and good replacement products are therefore difficult to make and a lot of attention and effort is put into this.

## Hypoxic preconditioning

Different approaches to activate or stimulate the ASCs to increase their regenerative potential have been attempted including hypoxic preconditioning. Hypoxic preconditioning is defined by short term exposure to hypoxia being 0.5-5% O<sub>2</sub> (Kang et al., 2014).

Normally, cultivation of cells including ASCs are carried out under atmospheric oxygen tension (20% O<sub>2</sub>) (Kang et al., 2014). However, it is widely known that atmospheric oxygen tension is not even close to the oxygen tension within the stem cell niche, where a low oxygen environment is physiological to adult somatic stem cells (Yamamoto et al., 2013). The exact oxygen tension physiological to different stem cells might vary, depending on the distance between the cells and the oxygen-supplying vessel and also the metabolic rate of the cells in the tissue (Taylor, 2008). Biological hypoxia is defined by an oxygen demand exceeding the oxygen supply, and the cellular demand of oxygen can vary depending on tissue requirements at a given time point (Taylor, 2008). However, it has been suggested that an oxygen level of 2-8% is a key aspect of the mesenchymal stem cell niche and thereby physiological to the stem cells (Mohyeldin et al., 2010).

The *in vitro* effects of hypoxic preconditioning of ASCs have shown to be increased proliferation of ASCs (Kakudo et al., 2015; Lee et al., 2009; L. Liu et al., 2013; L. Pilgaard et al., 2009; Rasmussen et al., 2011; Thangarajah et al., 2009), increased potency to form colonies (L. Pilgaard et al., 2009), and enhanced survival of the ASCs by reducing the apoptotic rate (L. Liu et al., 2013; Stubbs et al., 2012). Additionally, hypoxic culture have shown to maintain ASCs in an undifferentiated state without decreasing their differentiation potential (Lin et al., 2006). Additionally, conditioned media from hypoxic cultured ASCs have shown to increase endothelial proliferation, sprouting and tube formation and decreasing the apoptotic rate *in vitro* (Hollenbeck et al., 2012; L. Liu et al., 2013; Rasmussen et al., 2011; Rehman et al., 2004). It has also shown to increase fibroblast migration and collagen I secretion (Lee et al., 2009), and to increase the proliferation of keratinocytes (Park et al., 2010).

The *in vivo* effects of hypoxic preconditioning of ASCs have, amongst others, been found to be increased wound healing (Lee et al., 2009) and increased skin flap survival due to increased angiogenesis (Hollenbeck et al., 2012). Furthermore, hypoxic preconditioned ASCs was found to migrate deep into the tissue in a diffuse pattern, whereas non-conditioned ASCs localized around already existing larger vessels (Hollenbeck et al., 2012).

It is thought, that hypoxia might increase the regenerative potential of ASCs by increasing the secretion of different known pro-regenerative growth factors such as VEGF and bFGF (Hollenbeck et al., 2012; Kakudo et al., 2015; Lee et al., 2009; L. Liu et al., 2013; Rasmussen et al., 2011; Stubbs et al., 2012; Thangarajah et al.,

2009). By secreting these factors, the cells try to restore the oxygen supply by increasing the rate of angiogenesis (Ebbesen et al., 2004; Stubbs et al., 2012). The exact molecular mechanism behind hypoxia increasing the regenerative potential of ASCs is still not fully understood, but the major regulator is believed to be hypoxia-inducible factor 1 (HIF-1) which has been shown to be involved in the regulation of a plethora of genes and later proteins in ASCs (Kakudo et al., 2015; Kang et al., 2014; Song et al., 2010).

### **Characterization**

The International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) have proposed a set of guidelines for the characterization of ASCs (Bourin et al., 2013). These include testing of the viability by either flow cytometry or microscopic inspection. More than 90% of the ASCs should be viable. The proliferation and frequency should be tested using a CFU-F assay showing a frequency of more than 5%. Additionally the trilineage potential should be tested using histochemistry, reverse transcription polymerase chain reaction (RT-PCR), Western blot or ELISA. For the testing of this they have suggested a row of markers and stains to use including oil red O for lipid inclusions during adipogenic differentiation, alcian blue for staining glycosaminoglycans in cartilage and alizarin red for staining of calcium depositions during osteogenic differentiation (Bourin et al., 2013). All these elements characterize the identity of ASCs but not their regenerative potential and potency which in a clinical perspective probably even more important.

### **Potency testing**

The European Medicines Agency (EMA) have made a reflection paper on stem cell-based medicinal products including terminally differentiated cells derived from stem cells, undifferentiated stem cells or a mixture of stem cells composing different differentiation profiles (Committee for Advanced Therapies, 2011). They suggest that the potency of the stem cells should be tested in relation to the scientific rationale for the medicinal product based on the biological or cellular mechanism of action. Additionally, this should be tested using one or more potency assays comprising functional tests and marker-based assay. These assays should show correlation with the intended therapeutic use and be at least semi-quantitative. As examples of such assays they mention the expression of relevant biological substances, cell interactions and migration capacity (Committee for Advanced Therapies, 2011).



## CHAPTER 2. AIM, OBJECTIVES, AND HYPOTHESES

The aim of this dissertation was to aid the translation of ASCs into clinical use in wound healing.

In order to achieve this aim, this dissertation had two major objectives:

1. To identify the mode of action of ASCs in wound healing and how this is affected by hypoxic culture.
2. To identify how *in vitro* procedures of isolation and expansion affect ASC properties.

The hypotheses were:

1. ASCs play a role in wound healing through a paracrine stimulation of re-epithelialization, and this effect is enhanced through hypoxic exposure.
2. The establishment and expansion conditions are critical for developing clinically relevant ASC-based therapies.

To address the study aims and test the hypotheses, four different studies were conducted:

1. Hypoxia augments the wound healing effects of adipose-derived stem cells in primary human keratinocyte scratch assay.
2. Proteome analysis of adipose-derived stem cells cultured under clinically relevant conditions in a wound healing perspective.
3. Comparative Analysis of Media and Supplements on Initiation and Expansion of Adipose-derived Stem Cells.
4. Comparative Analysis of Subpopulations of Adipose-derived Stem Cells.



# CHAPTER 3. MATERIALS AND METHODS

A description of all methods and materials used in the studies described in this thesis are given in the individual papers. In this section, choice of materials and methods will be elucidated more in depth, explained or discussed wherever found relevant.

## 3.1. DONORS

To obtain ASC for subsequent analysis, different approaches were used throughout the thesis depending on the aim of the individual studies. When studying the response of ASCs to different stimuli, previously isolated, well characterized ASCs from three donors were used (ASC 12, 21, 23). In contrast, when studying the effect of isolation and early expansion protocols on the ASCs, freshly isolated ASCs were used (ASC 1-5, 101). Unfortunately, these could not be obtained from the same donors as the above mentioned 3 cell populations (Table 1).

*Table 1. Donor information.*

Donor no.	Sex	Age	Location	BMI	Fresh vs. Frozen	Paper
1	Male	46	Abdomen, hip, chest	26.2	Fresh	3
2	Female	53	Abdomen, thigh	21.5	Fresh	3
3	Female	62	Thigh	22.7	Fresh	3
4	Female	52	Arm	25.4	Fresh	3
5	Female	28	Knee, thigh	25.8	Fresh	3
12	Female	58	Back, hip, loin	28.0	Frozen	2
21	Male	52	Abdomen	n.a. <sup>1</sup>	Frozen	1, 2
23	Female	42	Abdomen, thigh	20.0	Frozen	2
101	Male	44	Abdomen	25.4	Fresh	4

<sup>1</sup>Information not available; BMI, body mass index

The previously isolated ASCs were after isolation and selection through a few passages stored at -140 °C until used for the studies in this thesis. The cells were washed and re-suspended in 37 °C culture medium before being seeded for further expansion and subsequent experiments. All used cultures were utilized before passage 8. Studies have shown, that ASCs maintain their proliferative capacity and stemness even after long term storage (Devitt et al., 2015; Minonzio et al., 2014), and therefore, using frozen cells were not giving rise to concern in this context.

To obtain fresh ASCs, adipose tissue was received from Teres Hospitalet Aarhus donated by patients undergoing elective liposuctions. The adipose tissue was stored at room temperature until processed. To isolate the ASCs from the adipose tissue, this was washed in phosphate-buffered saline (PBS) until the amount of blood remnants was minimized. At this stage, the adipose tissue was enzymatically digested with 0.005 g/mL collagenase (Collagenase NB 4, SERVA electrophoresis GmbH, Heidelberg, Germany) in Hanks Balanced salt solution (Gibco™) for 45 min at 37 °C under continuous vertical rotation. The digested adipose tissue was filtered through a 100 µm filter (Steriflip, Millipore) to remove non digested tissue lumps and centrifuged for 10 min at 400 g at room temperature. Afterwards the supernatant was discarded to remove adipocytes and the pellet was resuspended in PBS. This was then filtered through a 60 µm filter (Steriflip, Millipore) and centrifuged for additional 10 min at 400 g at room temperature. The supernatant was discarded and the pellet now constituted the SVF. The SVF was re-suspended in culture medium and the number of cells counted. If the cells were to be directly used for flow cytometric analysis the erythrocytes were lysed using distilled water. Otherwise, the SVF was seeded onto tissue culture polystyrene flasks (Greiner Bio-One) for selection of the plastic adherent ASCs. After incubation and washing, to remove non-adherent cells, the ASCs were used for subsequent analysis.

### 3.2. CULTURE OPTIMIZATION

The choice of culture medium varies between the studies in this thesis (Table 2). When ASCs were first discovered by Zuk (Zuk et al., 2001) Dulbecco's modified Eagle medium (DMEM) supplemented with FCS was recommended for culture. However, our laboratory tested alternatives and found that alpha-Minimum Essential medium (a-MEM) supplemented with FCS supported the growth of ASCs to a higher degree (Lund et al., 2009), and based on this, a-MEM has been used ever since in our laboratory as basal medium. In general, the majority of the studies of ASCs and their characteristics have been based on ASCs cultured in FCS supplemented media (Riis et al., 2015). However, when aiming for clinical use of the ASCs, the use of FCS has disadvantages. FCS is a complex mixture of known and unknown essential nutrients and bioactive molecules, it has high lot-to-lot variability due to its natural production method and the use it has given rise to concerns about the risk of contaminants and immunization due to the presence of xenogeneic components (Bal-Price and Coecke, 2011; Cholewa et al., 2011; Hemeda et al., 2014; Kølke et al., 2013; Shih et al., 2011). To overcome these limitations much attention has been and still is paid to the development of a fully defined serum-free alternative. In line with this, our laboratory started collaboration with LifeTechnologies to test a fully defined alternative, StemPro. Studies showed that this alternative was able to compete with the used of FCS in terms of supporting the ASC cultures and could be approved for clinical use under GMP regulations (Yang et al., 2012). Additionally, this choice of medium was if combined with the right supplement found compatible with subsequent mass spectrometry (MS)

analysis. However, later studies identified that StemPro did not support the initiation of ASC cultures (Study 3, Appendix 3), which also has been noted by others (Patrikoski et al., 2013). Another alternative to FCS is human platelet lysate (hPL), which contains a wide range of growth factors, proteins, enzymes supporting attachment, growth, and proliferation of cells, while still being poor in antibodies. It is isolated from common platelet units by a simple freeze-thaw procedure and has shown to be a safe alternative (Doucet et al., 2005; Rauch et al., 2011). When used as medium supplement, hPL has been shown to promote the growth of ASCs and maintain their differentiation potential (Doucet et al., 2005; Juhl et al., 2016; Li et al., 2015; Trojahn Kølbe et al., 2013; Witzeneder et al., 2013). The disadvantages of using hPL are that it is not fully defined, lot-to-lot variations do exist, and there is a small risk of pathogen carryover. Until fully-defined serum- and xeno-free alternatives are available, hPL looks like a good alternative to FCS. Based on this the effects of hPL was investigated in study 3 (Appendix 3).

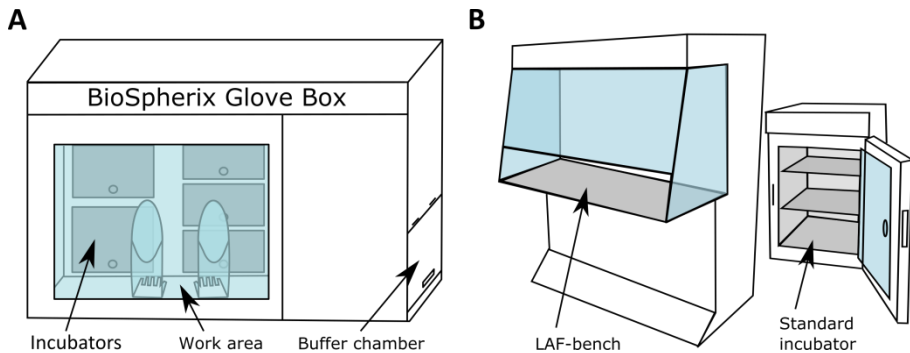
*Table 2. Media and supplement combinations used to culture ASCs.*

Media	Suppl.	Abb.	Coat.	Init.	Exp.	GMP	MS
alpha-Minimum Essential medium w. GlutaMAX	FCS	a-MEM <sup>FCS</sup>	-	+	++	-	-
StemPro MSC	StemPro MSC SFM XenoFree Supp.	StemPro or StemPro+	+	-	+++	++	-
Dulbecco's modified Eagle medium w. GlutaMAX	hPL	DMEM <sup>hPL</sup>	-	+	+++	+	-
alpha-Minimum Essential medium w. GlutaMAX	hPL 5 or 10%	a-MEM <sup>hPL</sup> <sub>hPL5</sub> or <sub>hPL10</sub>	-	+	++++	+	-
StemPro MSC	-	StemPro-	+		-	++	+
StemPro MSC	Essential 8	StemPro E8	+		-	++	+

*Abbreviations: Suppl., supplement; Abb.: abbreviation used in studies; Coat., coating; Init.: ability to support initiation of ASC culture; Exp.: ability to support expansion of ASC culture; GMP: compatibility with good manufacturing practice regulations; MS, compatibility with mass spectrometric analysis, FCS, fetal calf serum, hPL, human platelet lysate.*

### 3.3. HYPOXIC PRECONDITIONING

To study the effect of hypoxic preconditioning on ASCs, their paracrine response, and their wound healing properties a BioSpherix glove box (Xvivo, BioSpherix, Redfield, NY, USA) was used. This glove box consists of a buffer chamber, integrated incubators, and a work area (Figure 4A). The buffer chamber ensures maintenance of temperature and a constant gaseous environment inside the system. The integrated incubators enable long term culture within the system in a humidified environment corresponding to that of a standard incubator (Figure 4B). The work area enables manipulation of cells without removing them from the hypoxic conditions.



*Figure 4. Incubator systems used for preconditioning. A: BioSpherix glove box with separate incubators and a working area for hypoxic preconditioning. B: Standard incubator and laminar air flow (LAF) bench for standard culture and normoxic preconditioning. Modified from (Riis, 2012).*

The advantage of using the BioSpherix glove box compared to other hypoxic incubators is especially the work area by which the cells avoid being exposed to atmospheric air at any time point and thereby re-oxygenation. Re-oxygenation has shown to increase the intracellular levels of cellular oxidants including reactive oxygen species (Kim et al., 2007). Additionally cyclic hypoxia and re-oxygenation has shown to induce functional changes in cells including the resistance towards apoptotic triggers (Weinmann et al., 2004). This might be due to dysregulation of the cell cycle G2/M checkpoint, which has been shown to delay cell-cycle progression and promote the repair of DNA damage after re-oxygenation (Kim et al., 2007).

The choice of oxygen tension for the hypoxic preconditioning has been widely investigated by our laboratory, and 1% O<sub>2</sub> showed to give rise to the largest degree of secretion of proangiogenic factors (Rasmussen et al., 2011). Other oxygen levels have been suggested to promote other aspects, for example 5% O<sub>2</sub> have shown to promote the proliferation of ASCs to the largest extend. The choice of oxygen tension therefor depends of the aim of the preconditioning (Rasmussen et al., 2011).

### 3.4. CONDITIONED MEDIA

To study the paracrine response of ASCs conditioned media were produced by washing the cells in PBS and culturing them in fresh culture medium for 24 hours under either normoxic or hypoxic conditions (Figure 4). Hereafter, the conditioned medium was harvested, centrifuged to pellet cell debris and frozen at -80 °C for storage until further analysis.

For production of conditioned media different preconditioning periods are used throughout the literature (Hsiao et al., 2013). The length of the period might depend on the subsequent use of the conditioned media. If this is to be tested on another cell culture, the ratio between the secretory rate of the ASC and the ASC consumption of nutrients in the medium should be as large as possible. This is to ensure detectability of the effect of the secreted proteins, while still supporting cell maintenance of the target cells e.g. keratinocytes or endothelial cells.

### 3.5. CHARACTERIZATION

ASCs are recommended to be characterized by their immunophenotype, colony forming potential and differentiation potential (Bourin et al., 2013). However as more functional tests are recommended by EMA, these were added to our characterization (Committee for Advanced Therapies, 2011).

#### Immunophenotypic analysis

It is recommended by the IFATS and ISCT guidelines to test the immunophenotype of the ASCs by flow cytometry. More than 80% of the culture should be positive for CD13, CD29, CD44, CD73, CD90, and CD105. Furthermore, CD34 should be positive, but the levels may vary between donors. Additionally less than 2% should be positive for CD31, CD45 and CD235a (Bourin et al., 2013). To include all of these markers, simultaneously staining and analysis is not possible due to technical limitations of the flow cytometers available on the market today. To circumvent this, people normally analyses the presence of the markers one at a time and can therefore not predict the complete co-expression patterns. As we wanted to analyze the subpopulations of the ASCs as defined by their co-expression of surface markers, we found it necessary to choose a subset of the markers based on the interesting observations reported in the literature. This was well knowing that we did not include all of them.

To investigate the immunophenotype of the ASCs flow cytometric analysis was used applying a MOFLO Astrios EQ (Beckman Coulter). The analysis was based on a six color stain setup including anti-CD73 FITC on channel 488-513/26, anti-CD90 PerCP-Cy5.5 on channel 488-710/45, anti-CD105 APC on channel 640-664/22, anti-CD146 PE-CF594 on channel 561-614/20 and anti-CD271 PE-Cy7 on channel 561-795/70. Additionally, cell viability was assessed using a Live/Dead Fixable Aqua Dead Cell Stain (Molecular Probes, Taastrup, Denmark) on channel 355-448/59.

Fluorescence Minus One (FMO) controls were included for each fluorochrome, donor, and passage. Data were analyzed using Kaluza version 1.3 (Beckman Coulter). First, cells were gated on a forward scatter (FSC) / side scatter (SSC) contour plot to remove signals from noise and debris. For doublet discrimination a FSC (Width) / FSC (Height) contour plot was used. The non-viable cells were gated out based on the viability stain. The resulting cells were plotted in histograms corresponding to the above described markers, and overlays between FMOs and the stained sample were created. Positivity was defined by an overlay-marker, with a lower boundary set to include the top 2.5% of the FMO.

### Transcriptomic analysis

To assess the transcriptional level of differentiation markers in ASCs real time RT-PCR was used. ASCs were lysed and total RNA extracted using an Aurum™ Total RNA Mini Kit (Bio-Rad). cDNA was generated using an iScript cDNA Synthesis Kit (Bio Rad). To quantitate the original number of mRNA copies coding for the distinct markers real time RT-PCR was performed using primers for peroxisome proliferator activated receptor gamma (PPARG), osteocalcin (OCN) and SRY-box 9 (SOX9)(DNA Technology A/S)(Table 3), iQ SYBR Green Supermix (Bio-Rad), and a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The relative expression of each gene was calculated using a serial dilution of cDNA and subsequently normalized to the geometric mean of the expression of the two reference genes cyclophilin A (PPIA) and 3-/tryptophan 5-monooxygenase activation protein (YWHAZ) (DNA Technology A/S). These two reference genes have previously been shown to be relatively stable expressed in human ASCs during hypoxic culture (Fink et al., 2008). Same method has previously been used to investigate the transcription of the pro-angiogenic growth factor VEGF after hypoxic preconditioning of ASCs (Rasmussen et al., 2012).

Table 3. Primer sequences.

Target	Forward Primer Sequence	Reverse Primer Sequence
PPARG	5'- TCA GGT TTG GGC GGA TGC -3'	5'- TCA GCG GGA AGG ACT TTA TGT ATG -3'
OCN	5'- GAG CCC CAG TCC CCT ACC C -3'	5'- GCC TCC TGA AAG CCG ATG TG-3'
SOX9	5'- TTC GGT TAT TTT TAG GAT CAT CTC G -3'	5'- CAC ACA GCT CAC TCG ACG ACC TTG -3'
PPIA	5'- TCC TGG CAT CTT GTC CAT G -3'	5'- CCA TCC AAC CAC TCA GTC TTG -3'
YWHAZ	5'- ACT TTT GGT ACA TTG TGG CTT CAA -3'	5'- CCG CCA GGA CAA ACC AGT AT -3'

*PPARG*, peroxisome proliferator activated receptor gamma; *OCN*, osteocalcin; *SOX9*, SRY-box 9; *PPIA*, cyclophilin A; *YWHAZ*, 3-/tryptophan 5-monooxygenase activation protein



## Proteomic analysis

In contrast to transcriptomics of ASCs which has been widely analyzed throughout the literature, the extent and complexity of proteomic analysis is increasing as the technology develops. Proteomics is the study of proteins in biological systems during specific biological events. Protein expression is very dynamic and most proteins undergo post-translational modifications (PTM), which drastically increase the complexity of the proteome. To identify the presence and the concentration of a single or a narrow range of proteins an enzyme-linked immunosorbent assay (ELISA) can be applied as for VEGF or HIF-1 $\alpha$  (Rasmussen et al., 2012). However, to identify new proteins in a broader perspective or look deeper into the plethora of proteins synthesized and secreted by ASCs a more discovery based strategy must be used as for example MS. This technology is based on the detection of mass-to-charge ratio ( $m/z$ ) of ionized molecules in simple or complex mixtures. It can be used to study proteomics in terms of detection, identification and quantification of peptides and proteins by matching the masses and charges of these to different databases. This approach allow for the identification and relative quantification of almost all expressed proteins of complex organisms.

To assess the effect of hypoxic preconditioning on the proteome and secretome of ASCs, samples for MS analysis was prepared (Figure 5). For this, ASCs from the three well characterized donors were cultured and preconditioned under normoxic and hypoxic conditions as described above. The only difference was that the cells were conditioned in StemPro basal media supplemented with E8 supplement due to technical limitations of using the original StemPro supplement for MS analysis. After being expanded for 72 hours and preconditioned for 24 hours, the conditioned media were harvested and protease inhibitors were applied to avoid excessive degradation of proteins by naturally occurring proteases. After this the conditioned media were spun down to remove cellular debris. Additionally, the ASCs were harvested using a RIPA buffer for proteome analysis. All samples were stored at -80 °C until further analysis.

To analyze the proteins secreted by the ASCs into the medium the conditioned media were fractionated into two fractions by using cut of spin filters, one fraction above 30 kDa and one below 30 kDa. The proteins of the two fractions were extracted using a sodium deoxycholate based protein extraction protocol, reduced and alkylated followed by an in solution trypsin digestion.

To analyze the content of the proteome fraction, this was reduced and run on a polyacrylamide gel in one dimension according to molecular weight for subsequent in-gel digestion by trypsin.

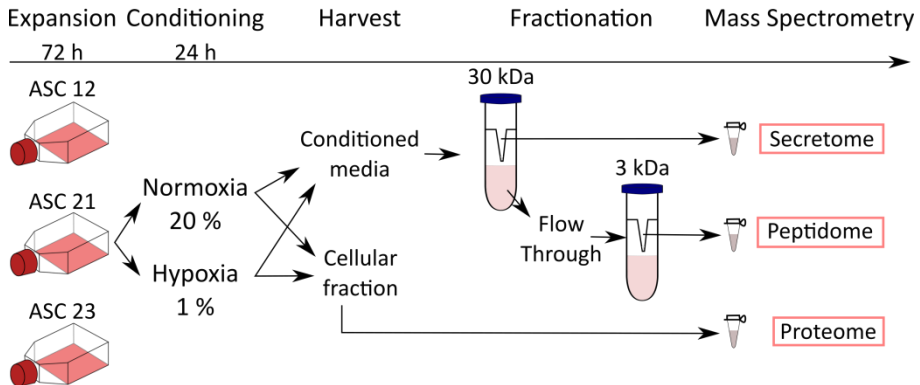


Figure 5. Sample preparation for mass spectrometric analysis. ASCs from three donors were expanded, preconditioned, and the conditioned media and cellular fractions harvested. The conditioned media were fractionated into two fractions; one above 30 kDa and one below 30 kDa. The secretome, peptidome and proteome were subsequently analyzed by mass spectrometry. Abbreviations: ASC, adipose-derived stem cell; h, hours; kDa, kilo Dalton.

The digested samples from all fractions were loaded onto a liquid chromatography column and sequentially eluted to ensure separation of the peptides and increase the peptide concentration. The peptides were continuously ionized by electrospray ionization (ESI) to form gas-phase ionized peptides. These were then introduced to the MS by vacuum. The ionized peptides were manipulated in the mass analyzer using electrostatic fields which deflect the paths of individual ions based on their mass and charge ( $m/z$ ) to separate and filter the peptides. The peptides were detected by an ion detector measuring mass-to-charge ratio ( $m/z$ ) of the deflected ionized peptides. The data from the ion detector were subsequently analyzed by MaxQuant and Perseus software. The proteins were identified by matching the peptide intact masses and measured fragment  $m/z$ 's against a database. To quantitate the proteins a label-free quantification approach based on the correlation of signal intensity and peptide abundance was used. See Figure 6 for an overview of the MS process.

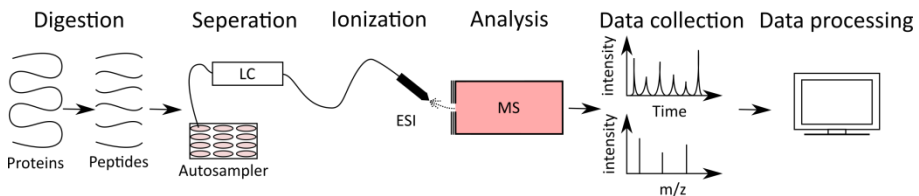


Figure 6. Principle of the protein analysis by mass spectrometry (MS). The proteins in the sample are digested into peptides. These are loaded onto a liquid chromatography (LC) column using an auto sampler. As the peptides are sequentially eluted they are ionized by electrospray ionization (ESI) and injected into the mass spectrometer (MS). Subsequently the peptides are analyzed for time of injection, mass and charge ( $m/z$ ) and the data processed to identify the original proteins.

### 3.6. FUNCTIONAL ASSAYS

For clinical use, one aspect is the identity of the ASCs, another important aspect is their mechanism of action and potency towards relevant aspects of the intended use (Committee for Advanced Therapies, 2011). In our case, we intend to use the ASCs for wound healing and based on that we were looking into the effect of ASCs on different aspects of this.

#### Re-epithelialization

To mimic the re-epithelialization during wound healing different models were investigated.

A 3D-model was of special interest as this mimicked the *in vivo* situation the most compared to other alternatives. This model is based on the seeding of primary keratinocytes on filter inserts, on which the cells are exposed to differentiation medium while being air-lifted. This model is widely used in other research areas and has been shown to histologically be representable for the epidermis of the skin (Poumay et al., 2004). However several practical issues did, that the final choice became a keratinocyte based monolayer scratch assay.

Before the re-epithelialization model could be established different aspects had to be optimized. Among the simpler were choice of coating, seeding density of cells, time of wounding, and wounding method. The wounding method should be simple and reproducible. The most used method is the use of a pipet tip to scratch a monolayer of cells (Liang et al., 2007). This method is the most inexpensive, however it is not easy to handle and not very reproducible. A variety of “scratching pins” have been developed in an attempt to make the scratching more handleable, however it is still time consuming to manually scratch each well and the placement of the scratch within the well is still difficult to reproduce. The Wounding Pin Tool (V&P Scientific, INC.) circumvents this by scratching up to 96-wells simultaneously with matching placements of each scratch. The WoundMaker™ (Essen BioScience) has the same advantages, but on top of this, it also controls the force by which the scratches are created and makes this reproducible between experiments. Based on this the two latter were used in Study 1 for keratinocytes and in Study 4 for endothelial cells.

A more challenging aspect of the assay was the preceding production of conditioned media for testing on the assay. The goal was to investigate the effect of hypoxic preconditioning on the secretome of the ASCs. However, directly testing of ASC conditioned media on primary keratinocytes showed impossible due to the fact that the calcium levels in StemPro used for production of conditioned media were incompatible with the maintenance of primary keratinocytes. Different strategies to overcome this were tested and the most successful approach was dialysis of the conditioned media into EpiLife medium normally used for keratinocytes before testing it on the assay. This ensured normal keratinocyte morphology and wound

closure of the scratch when compared to standard culturing conditions for primary keratinocytes.

For the testing of the conditioned media primary keratinocytes were seeded at a density forming a confluent monolayer. After overnight incubation, the monolayer was scratched using one of the above mentioned scratching tools and washed in PBS to remove cell debris from the wound edges. Media were added to the cells and wound closure monitored. Closure of the scratch was monitored by time-lapse microscopy taking phase contrast pictures every hour. Finally, wound size at each time point was compared to the size of the initial scratch, and the wound closure calculated.

The biggest limitation of using this assay is the simplicity. It is to mimic the epidermis, but the epidermis *in situ* is a multilayer structure, with interlayer differences in cellular organization and function. This could be optimized using the 3D skin model.

# CHAPTER 4. SUMMARY OF THE RESULTS

Detailed results are described in each manuscript (Appendix 1-4). In this section a brief overview of the main findings will be given.

## 4.1. FIRST HYPOTHESIS

### **ASCs promote re-epithelialization and hypoxic preconditioning enhances this effect**

To investigate the effect of ASCs on re-epithelialization and whether hypoxic preconditioning of ASCs has a positive effect on this, a keratinocyte based scratch assay was established and the conditioned media derived from either normoxic or hypoxic conditioned ASCs were applied. When assessing cell morphology of the keratinocytes exposed to either the normoxic or the hypoxic conditioned media, no differences were observed. When assessing wound closure it appeared that the keratinocytes supplied with hypoxic conditioned media closed the scratch at a faster rate than the keratinocytes supplied with normoxic conditioned media (Appendix 1, figure 5A). When measuring wound size, it was evident that the keratinocytes supplied with normoxic conditioned media from ASCs closed the wound area at faster rate than the keratinocytes in control media, and that keratinocytes supplied with media from hypoxic conditioned ASCs closed the wound area even faster. This difference was statistically significant 24 hours after the scratch was made (Appendix 1, figure 5B).

### **Hypoxic preconditioning affects the proteome of ASCs**

To investigate the effect of hypoxic preconditioning on the overall proteome of ASCs MS analysis was applied (Appendix 2). To assess the effect of hypoxic preconditioning on the content of the conditioned media, the secretome of ASCs was analyzed and no difference was found between the proteins identified in normoxic and the hypoxic conditioned media.

To assess the effect of hypoxic preconditioning on the intracellular proteins of ASCs, the proteome of the normoxic and hypoxic preconditioned ASCs was analyzed. When looking into the identified proteins in the normoxic and hypoxic preconditioned samples and the reoccurrence between the three donors, it was found that the majority of the proteins were identified in all samples (Appendix 2, Figure 3A). When analyzing the differences between the samples, it was by a principal component analysis (PCA) found that the inter donor variation gave rise to a bigger degree of difference in the data than the effect of hypoxic preconditioning (Appendix 2, Figure 3B). Furthermore, the biological processes in which the effect

of hypoxic preconditioning was significant were analyzed. When mapping the genes, coding for the proteins found statistically significant upregulated by hypoxic preconditioning several enriched biological processes were identified including anaerobe metabolic processes and ECM remodeling relevant processes (Appendix 2, Figure 4 and Table 1). The latter included proteins involved in tensile strength, three-dimensional folding, and mechanical stability of collagen fibrils in the dermis ensuring skin integrity and stability.

When looking at the proteins that were down-regulated by the hypoxic preconditioning, the enriched biological processes were mainly related to gene expression and protein synthesis and aerobic metabolism indicating a switch in metabolism relevant proteins demonstrating a change from aerobic to anaerobic metabolism (Appendix 2, Figure 5 and Table 2).

## 4.2. SECOND HYPOTHESIS

### **The composition of culture medium influences the establishment of ASC cultures**

To assess how the composition of culture medium influences the establishment of ASC cultures, a variety of widely used medium compositions were tested on culture initiation (Study 3, appendix 3). The media were tested in terms of how they initially supported the plastic adherence of ASCs, the growth of ASCs in the expansion phase and during this also the selection of specific subpopulations within the ASCs (Figure 7).

To evaluate the ability of the different media compositions to support the plastic adherence of ASC, freshly isolated SVF and early passage ASCs were used. After the isolation and seeding of the SVF and overnight culture, the non-adherent cells were removed and attached to the plastic surface were, for all media types, a morphological mixed population of mostly spheric and a few slightly elongated cells (Figure 7A). After passaging the SVF to obtain ASCs a more homogenously looking population began to arise with larger looking and more spindle shaped cells. When counting the number of cells attached to the surface it was evident that StemPro supported cell attachment to a larger degree than the other medium compositions and especially the hPL supplemented solutions. In contrast, DMEM supplemented with hPL did not support attachment to the same degree as any of the other medium compositions (Figure 7B).

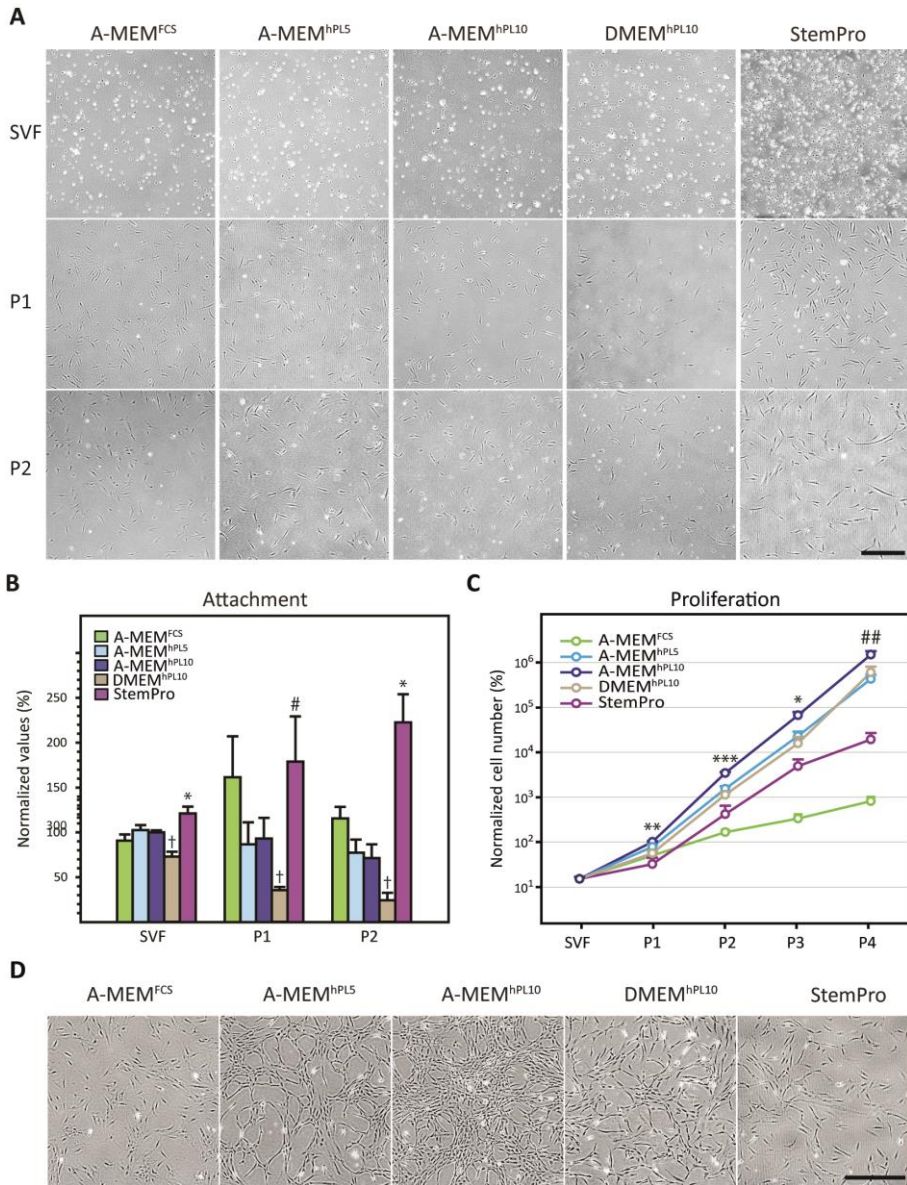


Figure 7. Attachment and proliferation of Adipose-derived Stem Cells. A: Differences in the degree of cell attachment was observed after seeding and overnight incubation. Additionally morphological differences were seen between passages. B: Quantification of cell attachment revealed significant differences between media compositions. † statistically different from all other media types with  $p < 0.05$ , \* StemPro statistically different from all other media types with  $p < 0.05$ , # StemPro statistically different from all other media types except A-MEM<sup>FCS</sup> with  $p < 0.05$ . C: Accumulative cell number after a series of passages, where a clear effect of

the media is shown. \* A-MEM<sup>hPL10</sup> statistically different from all other media types with  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ## A-MEM<sup>hPL10</sup> statistically different from all other media types except DMEM with  $p < 0.007$ . D: ASC cultures reached different cell densities during expansion depending on the media type, as evident at passage 1 after four days of culture. The results are presented as mean  $\pm$  SEM. The cell number is normalized to the cell number of each individual donor for A-MEM<sup>hPL10</sup> at passage 1. The scale bar depicts 500  $\mu$ m. Abbreviations: A-MEM, alpha-Minimum Essential Medium; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; hPL, human platelet lysate; SVF, stromal vascular fraction; P, passage. Reprinted with permission from (Study 3).

To study the effect of medium composition on cell growth, freshly isolated SVF were cultured for up to three passages in the different medium compositions. When assessing the cultures under a microscope it appeared that the hPL supplemented media supported the growth of ASCs to a larger degree than both StemPro and medium supplemented with FCS (Figure 7D). This was confirmed by counting the cells, where a significant higher cumulative cell number was found in all media supplemented with hPL than in the other media (Figure 7C). Interestingly, StemPro did not consistently support cell growth, and for some donors, the cell cultures in StemPro could not be satisfactorily maintained for more than three passages.

To study the effect of the different medium compositions on the selection of specific subpopulations within the heterogeneous ASC population, the cells cultured in the different media were analyzed by flow cytometry and the co-expression of surface markers at three time points during the expansion phase determined (Figure 8). After isolation the SVF consisted of a very heterogeneous composition of nearly all combinations of the surface markers investigated. However, the majority of the cells were CD90<sup>+</sup>, CD105<sup>+</sup>, CD146<sup>+</sup>, CD271<sup>+</sup>, and then either CD73<sup>+</sup> or CD73<sup>-</sup>. After the SVF had been passaged once, differences in the cell composition of the populations cultured in the different media began to appear with the StemPro cultures retaining their heterogeneity and the FCS/hPL supplemented cultures becoming more homogenous. The majority of the cells cultured in these media were now CD90<sup>+</sup>, CD105<sup>+</sup> and either CD73 positive or negative, and the CD105<sup>-</sup> subpopulation had disappeared (Figure 8).

After seven passages the surface marker profile of the populations in the different media compositions was analyzed once more. However, none of the StemPro cultures made it for seven passages and could therefore not be analyzed. On the other hand, in the remaining cultures the CD73 negative subpopulations were no longer found, making the cultures even more homogenous than after the first passage. For all donors, the majority of the cells in the different media were now found to be CD73<sup>+</sup>, CD90<sup>+</sup>, CD105<sup>+</sup>, CD271<sup>+</sup> and either CD146<sup>+</sup> or CD146<sup>-</sup>. Summarizing, no major difference between A-MEM and DMEM as choice of basal media or FCS or hPL as choice of supplement was identified when assessing the composition of the subpopulations (Figure 8). However, inter-donor variations were found in the relative size of the CD146 positive and negative populations.



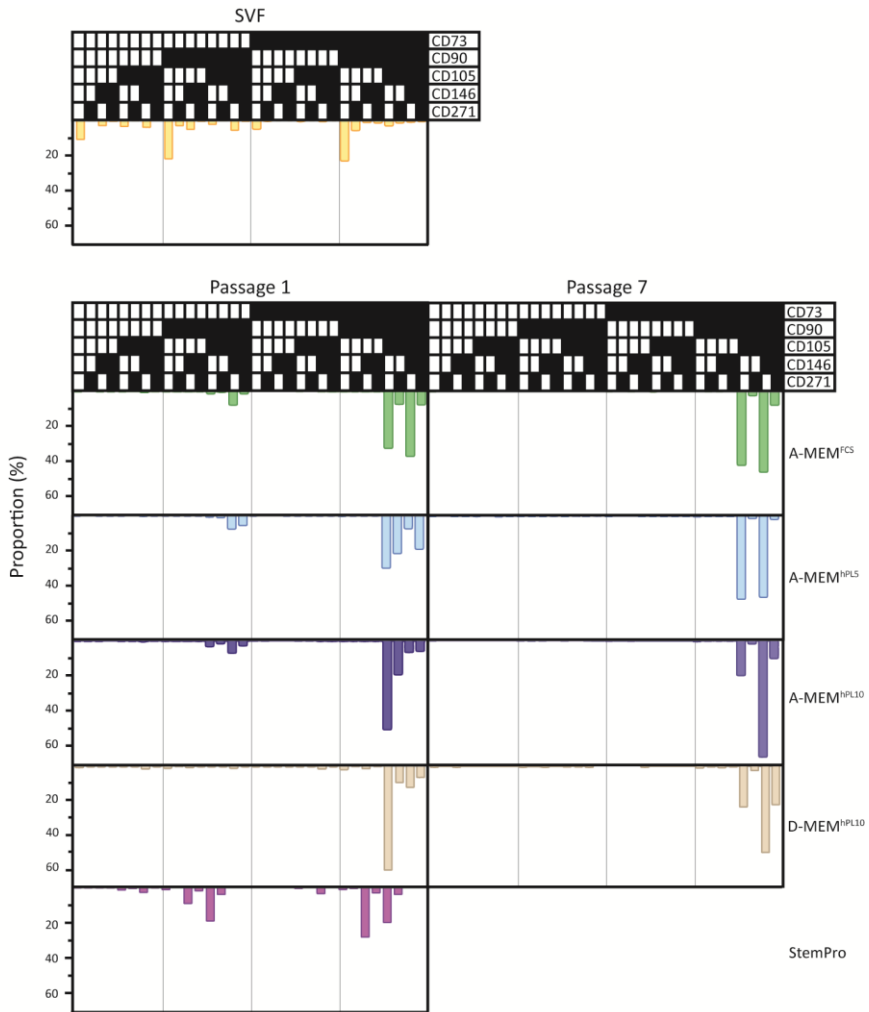


Figure 8. Co-expression of surface markers on SVF and ASCs. SVF showed very heterogeneous. After 1 passage, ASCs became more homogeneous despite media composition. Homogeneity was even larger after 7 passages. Overall, subpopulations had the same characteristics, but differences were found in the relative size of the different subpopulations. Representative data are compiled from two donors. ■ the surface marker is expressed, □ the surface marker is not expressed. Abbreviations: SVF, stromal vascular fraction; FMO, fluorescence minus one; A-MEM, alpha-Minimum Essential Medium; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; hPL, human platelet lysate. Modified and reprinted with permission from (Study 3).

### **The distinct subpopulations within the ASC population have different properties**

To assess whether the different subpopulations within the heterogeneous composition of ASCs have different characteristics, SVF was sorted into four subpopulations based on their surface marker profile using flow assisted cell sorting. Subsequently, the crude ASC population and the subpopulations were characterized in terms of stem cell characteristics and their regenerative potential (Appendix 4).

To study the maintenance of the subpopulations during *in vitro* expansion each subpopulation was seeded into culture flasks and expanded for 3 passages. After 2 and 3 passages the surface marker profile of each population was analyzed. The analysis revealed that just two passages after sorting, the surface marker profile of all four subpopulations had moved towards the composition of the crude ASCs by regaining the profiles originally discarded by sorting. The same pattern was found after 3 passages. However, subpopulation 2, originally CD34<sup>+</sup>, CD146<sup>+</sup>, CD271<sup>-</sup>, maintained a higher fraction of the originally sorted marker profile for all analyzed passages. Additionally, this subpopulation had a higher fraction of CD34<sup>+</sup>, CD146<sup>+</sup>, CD271<sup>-</sup> cells compared to all the other populations (Appendix 4, Figure 4).

To evaluate the differentiation potential of the subpopulations these were induced towards adipogenic, chondrogenic and osteogenic differentiation. When assessing the subpopulations under a microscope all showed capable of differentiating into the three lineages as did the crude ASCs. This finding was confirmed by real-time q-PCR where no difference between the subpopulations was found (Appendix 4, Figure 5). To measure the proportion of clonogenic cells in each population of either the crude ASCs or the subpopulations, these were analyzed after two passages using a limiting dilution based CFU assay (Appendix 4, Figure 5). No difference was found between any of the populations.

To evaluate the regenerative potential of the crude ASCs and the different subpopulations, the ability of each population to support an endothelial-based scratch assay was investigated by transferring conditioned media from the distinct populations to the assay. This showed that the crude ASC population and the subpopulations in general supported endothelial-based wound closure and moreover, that subpopulation number 2 increased the speed of closure significantly more than any of the other subpopulations (Appendix 4, Figure 6).

## CHAPTER 5. GENERAL DISCUSSION

ASCs have been shown to have wound healing properties, but as wound healing is a complex process of cellular, microenvironmental and molecular events, the exact mechanism of how the ASCs conduct their wound healing effect is difficult to clarify. Several attempts to map the mechanism of action have been made and many elements of the wound healing effect have been identified. However, the full explanation is still not clear.

### **First hypothesis**

ASCs play a role in wound healing through paracrine stimulation of re-epithelialization and this effect is enhanced through hypoxic exposure.

In Study 1 it was shown that ASCs stimulates re-epithelialization. In an earlier study it was shown that ASCs stimulate angiogenesis through the secretion of VEGF and that this effect is enhanced by hypoxic preconditioning and trypsin treatment (Rasmussen et al., 2012). The indication of ASCs being a multi-target approach not only stimulating angiogenesis but also re-epithelialization seems highly valuable as chronic wounds per say encompasses a broad range of etiologies, and by targeting multiple facets of wound healing with the same intervention the probability of treatment success increases. Earlier, different strategies targeting single aspects of wound healing have been attempted without clear evidence of the general effect talking for the use of a multi-target approach (Mustoe, 2004; Sundhedsstyrelsen, 2011).

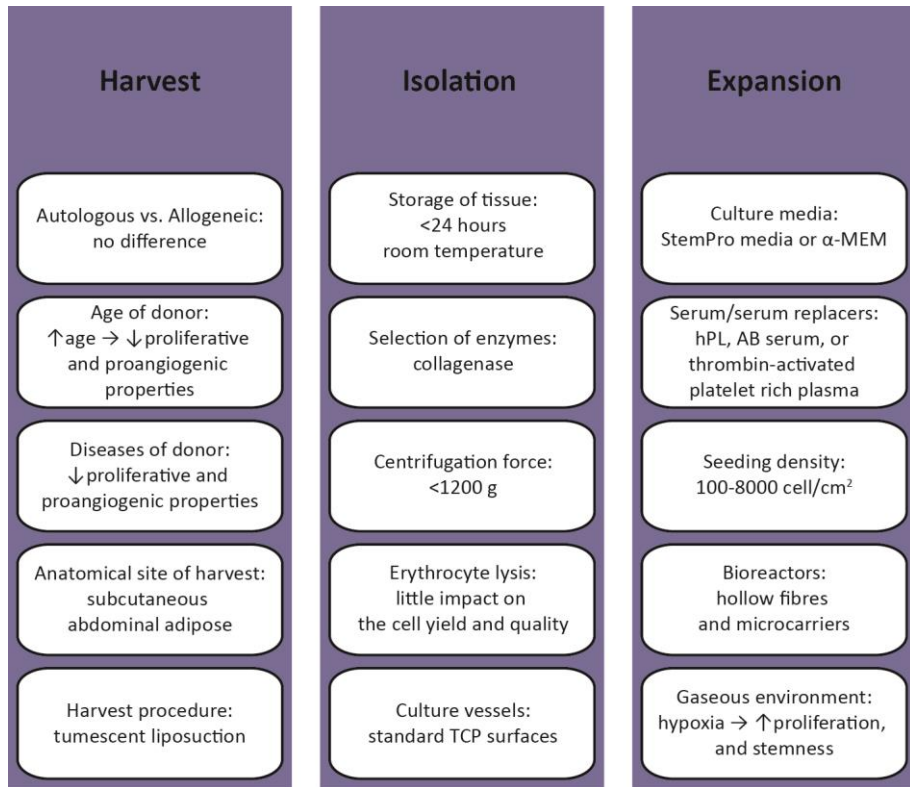
In Study 1 it was also found that the effect of ASCs on re-epithelialization was enhanced by hypoxic preconditioning of the ASCs and the same applies for angiogenesis where the effect of ASCs also was increased by hypoxic preconditioning (Rasmussen et al., 2012). Furthermore, that study showed that the effect of preconditioning could be even larger when combining hypoxia with trypsin treatment. The synergistic effect of hypoxia and chemical factors as trypsin on the transcription and secretion of e.g. VEGF may form the basis for a novel paradigm of preconditioning of ASCs prior to their clinical application. By not only using hypoxia but also other stimulating approaches to precondition the ASCs, a highly efficient stem cell based product could be obtained. Other laboratories have used other pre-conditioning strategies than hypoxia or trypsin to increase the regenerative potential of ASCs. These strategies include chemical stimulation using for example vitamin C (Kim et al., 2014), physical stimulation using culture surfaces with different geometry (Amos et al., 2010; J. Seo et al., 2014; Xia et al., 2014), ultra violet light (Jeong et al., 2013) or many other physical and biochemical strategies (Cavallari et al., 2012; Dongó et al., 2014; Hoke et al., 2012; Lee et al., 2015; G.-S.

Liu et al., 2013; Lu et al., 2013; Mehrabani et al., 2015). Additionally, it has been suggested that other gaseous messengers than oxygen might regulate stem cells in their natural niche, suggesting the investigation of the effect of other gases on the properties of stem cells (Mohyeldin et al., 2010). The significant effect of combining preconditioning factors must be investigated and evaluated against the gain in clinical effect, as adding another level of complexity to the production of a stem cell based product increases the level of GMP regulatory work.

In our studies both angiogenesis and re-epithelialization were stimulated through paracrine factors secreted by ASC into the conditioned media. Attempts to clarify the content of the conditioned media have been made and the factors identified so far have been tested on wound healing, nonetheless, no single factor or simple combination of factors have yet been found solely responsible for the wound healing effect of ASCs (Hassan et al., 2014; Rasmussen et al., 2011). This should be seen in the light of wound healing being a complex process involving a plethora of cytokines, chemokines and growth factors (Barrientos et al., 2008; Behm et al., 2012; Werner and Grose, 2003). Despite this, knowing the exact content of the media conditioned by ASCs would enable a large scale production of a treatment modality based of a mixture on growth factors, cytokines and other soluble factors making the mapping of the secretome very desirable.

In study 2 it was attempted to clarify the effect of hypoxic preconditioning on the composition of the secretome and the proteome of ASCs. The study showed that great donor variation in the secretome and proteome existed. This has also been identified by others (Kalinina et al., 2015). Additionally, it was shown that hypoxic preconditioning of ASCs affects their proteome by shifting from aerob metabolism processes to an anaerob metabolism. Moreover, proteins involved in ECM remodeling acting through regulation of the composition, alignment and mechanical properties of the ECM components were found upregulated. Furthermore, it was attempted to clarify the effect of hypoxic preconditioning on the content of the conditioned media. Even though it has been shown by us and others that ASCs do increase wound healing through a paracrine mechanism, we were not able to identify the factors responsible for this by the use of MS analysis. Others have tried to use the same strategy to clarify the effect of hypoxic preconditioning on the secretome of ASCs, however they did not find a measurable effect by this (Kalinina et al., 2015). Nevertheless, even though the content of the conditioned media was mapped and a full explanation of the wound healing effect identified, a chemically defined replacement of an ASC based therapy is unlikely to meet the need for a stem cell based product. Studies have shown, that ASC conditioned media alone cannot match the effect size of the ASCs, possibly explained by the ASCs responding to the environment around them and thereby changing their paracrine response (Kim et al., 2012; Mishra et al., 2012; Rehman et al., 2004; B. F. Seo et al., 2014). This dynamic mechanism will not be possible to replace with a commercial off-the-shelf product with a defined content of soluble factors. The use of such instead of injecting ASCs

into the wound bed will possibly decrease the general effectiveness towards the application of chronic wounds with its board and complex etiology.



*Figure 9. Overview of the critical steps in the harvest, isolation and expansion of adipose-derived stem cells (ASCs). For isolation and expansion of ASCs for translational therapy there are a lot of aspects to consider all affecting the end product. The process can be divided into three parts with each their aspects. First part is the harvest of the adipose tissue from which the ASCs shall be isolated. Here it is important to consider donor aspects such as auto- or allogenic, age and diseases and harvest aspects as technique and site to harvest from. For the next part, the isolation, the critical considerations are how to storage the tissue prior to isolation regarding time and temperature, selection of digestion enzymes for digestion of the adipose tissue effectively without affecting the cell integrity, centrifugal forces for effectively pelleting the stromal vascular fraction (SVF) without destroying the cells, erythrocyte lysis method to get a cleaner SVF and culture vessel for selection of ASC from the SVF. The third part is the expansion part where ASCs selected from the SVF are expanded to reach the therapeutic dose for the therapy of interest. For this part it is critical to choose the right culture media inc. supplements, seeding density and culture method, this being one layer, multi-layerd or bioreactor again depending on the therapeutic dose needed. Modified and reprinted with permission from (Riis et al., 2015).*

## Second hypothesis

The establishment and expansion conditions are critical for developing clinically relevant ASC-based therapies.

To clarify the effect of establishment and expansion of ASCs on their cellular properties a review was compiled to identify the critical steps in the isolation and expansion process of ASCs when targeting regenerative therapies as for example the healing of chronic wounds (Riis et al., 2015). From this it was evident, that many practical aspects regarding the isolation and expansion of ASCs are to be taken into consideration when designing an ASC based study and later on an ASC based treatment modality. In the review, the recommendations, based on studies investigating different aspects of the process, was gathered to give the reader an overview of which aspects has already been studied and which need further examination to increase the level of evidence. The main conclusions of the review are summarized below. For references please refer to the review (Riis et al., 2015). To provide a better overview of the process, we categorized the critical steps into either harvest, isolation, or expansion. To summarize the findings, the critical steps and the recommendations hereon are here presented in short (Figure 9). For the harvest, the critical steps include donor selection, the anatomical location of harvest and the actual harvest procedure. The ideal choice of donor is still widely discussed, and might be dependent on the application. In this context the big question is whether to use autologous or allogenic ASCs (See Chapter 1.4.). The site of harvest and harvest procedure has been thoroughly investigated and the recommendations are to use subcutaneous abdominal adipose tissue harvested by tumescent liposuction. For the isolation process it was clear that the protocol first suggested by Zuk (Zuk et al., 2001) is still the foundation of the protocols used worldwide, however with a wide range of optimizations, adjustments and clarifications based on different subsequent studies. Storage of tissue, selection of enzymes for enzymatic treatment and choice of culture vessel are among those aspects widely investigated. Last, gathering information about the expansion process made it clear that this is the part of the overall process towards which most attention has been payed. A wide variety of studies was found aiming towards identifying the best protocol for expansion of ASCs ensuring a high cell number while maintaining the stem cell characteristics. Additionally, an increasing interest in ensuring good manufacturing practice (GMP) was observed, making the protocols clinically relevant and retaining the application specific regenerative properties of the ASCs. In this context can be mentioned considerations about the choice of culture media, large scale productions based on bioreactor systems and also the gaseous environment. The latter have been shown to affect and possibly increase the stem cell characteristics when used properly.

Choice of culture medium was identified as an aspect still giving rise to a large amount of research and experimenting product development, where serum- and xeno-free candidates are making their entry onto the market. Studies have shown big

difference in the ability of different media compositions to support proliferation, maintenance of multipotency and immunological properties (Patrikoski et al., 2014). Thus, much attention has been paid to the effect on the stem cell characteristics as defined in IFATS and ISCT guidelines (Bourin et al., 2013; Dominici et al., 2006), and less on other, potentially more, clinically relevant aspects such as surface marker co-expression, cellular sub-type destiny, and clinical application specific potency assays.

In study 3 it was shown that the medium composition affects the isolation and expansion of ASCs both in terms of cell doubling time and the composition of subpopulations. The true significance of these findings is still unclear. The aspects as population doubling time have a direct practical value as shorter doubling time decreases production time and the expenses of a stem cell based product. However, the regenerative value of a faster proliferating population is still uncertain. On the other hand, the composition of subpopulations is difficult to rank as long as the function of each subpopulation is not fully understood as indicated through literature review by others (Baer, 2014). We found subpopulations defined by both the presence of specific surface markers and by the intensity of the markers. The question about function of subpopulations based on the presence/absence of specific markers has given rise to speculation (Blocki et al., 2013; Pierantozzi et al., 2015; Russell et al., 2010), but difference in intensity seems still sparsely studied. Differences in subpopulations, have broadly been suggested to give rise to differences in for example content of the secretome and thereby give rise to different efficiencies and side effects of ASCs (Kalinina et al., 2015).

In study 4 the characteristics and properties of four subpopulations were studied. It was shown that the subpopulations composing the heterogeneous ASCs did not differ in terms of stem cell characteristics indicating that no superior stem cell exist within the ASCs based on cell division and characterization. Nonetheless, one subpopulation seemed to be superior in the stimulation of endothelial cells. In line with this, others have shown differences in the immunomodulatory properties of ASC subpopulations (Sempere et al., 2014). However, even though we sorted a specific subpopulation for further analysis, the composition of surface markers returned to that of the crude ASCs by passaging. This indicates that one subpopulation cannot exist on its own but need the presence of the others whatever functions they contribute with. In this heterogeneous constellation each subpopulation might contribute with distinct functions giving crude ASCs it board panel of potential applications. In this light, the in study 3 found donor variations regarding the relative size of each subpopulation which also have been found by others (Baer et al., 2012), might indicate, that some donors poses a larger regenerative potential towards some applications than others, depending on their subpopulation composition. If a correlation once is identified between the presence and relative size of a certain subpopulation and a good clinical effect, this could be used as a screening tool to identify an ideal donor for that certain clinical

application. However it should be kept in mind that elements critical for the clinical use in towards condition might not be of same importance in another clinical application.

## 5.1. LIMITATIONS OF THE STUDIES

### First hypothesis

The choice of wound model in our study was very simplified. When using *in vitro* models, the cells, forming the basis for the model, are taken from their natural niche and exposed to conditions very far from the physiological ones. Evaluating the wound healing properties of the ASCs on such a model under these conditions might be misleading, as the cells normally residing in a wound might behave differently *in vitro* than they do *in situ*. Keratinocytes normally form a multilayered structure with basal proliferating keratinocytes and apically a cornified layer of keratinocytes which is far from our monolayer. Using a 3D model resembling the natural stratified structure would have increased the power of the findings. Another aspect could be to use keratinocytes isolated from chronic wounds as keratinocytes in and around chronic wounds reside in an impaired state and are not able to fully participate in the healing process as they have been shown to produce a decreased level of growth factors (Demidova-Rice et al., 2012). The effect of ASCs on this would be valuable to take into consideration. However, using *in vivo* models instead of *in vitro* ones might give a more accurate picture of the process and the effect of the intervention. Attempts to find a good animal model have been made, however major obstacles of investigating the healing of chronic wounds is the fact that animals do not naturally get chronic wounds but acute wounds must be inflicted and infection induced (Mustoe, 2004), and that rodents often used heal by other mechanisms than humans (Toyserkani et al., 2015). A full review of this has been made by Nunan et al. (Nunan et al., 2014). The best option is of course to use human patients suffering from naturally occurring chronic wounds with the vicious chronic wound environment (Thamm et al., 2013), but here ethical considerations and safety issues are to be dealt with to a much larger extend than when using animal models.

The choice of using MS analysis to investigate the content of the conditioned media had some disadvantages. First a culture medium both compatible with ASC maintenance and MS technology had to be identified. Normally cells are grown in a culture medium with a high abundance of albumin from serum or medium supplements (Francis, 2010), but this is not compatible with the MS technique as high abundant peptides can dominate the mass spectrum limiting the detection and subsequent analysis of lower abundant peptides (Hawkridge, 2014). To circumvent this, ASCs are normally serum starved for 24-72 hours before the conditioned media is to be harvested. However, serum starving the ASCs will induce stress to the cells that might obscure the effect of hypoxic preconditioning (Follin et al., 2013; Tratwal et al., 2015). To avoid this, we sought an alternative supplement not containing high abundant proteins as albumin but still supporting ASC maintenance. This was



partially found, but as it did not support expansion of the ASCs a switch in medium type before conditioning was still necessary, possibly affecting the cells in some way. To overcome this, a culture medium fully compatible with ASC maintenance and MS technology must be identified before the secretome can be fully explored.

Second, the sensitivity of the data analysis of the MS data towards very slight increases in cytokine concentration is unknown. By using ELISA we have found that by hypoxic preconditioning VEGF secretion into the medium was increased two-fold (Rasmussen et al., 2012). This was enough to cause a measurable biological difference on endothelial cells, but we could not detect this increase in VEGF by MS analysis. This might explain some of the reason why we could not measure any effect of hypoxic preconditioning on the secretome, and thereby our findings should not be used as an indication of no effect of hypoxic preconditioning on the ASC secreted proteins.

### **Second hypothesis**

When studying the effect of establishment and expansion of ASCs on their cellular properties inclusion of more donors would have added more power to the statistical analysis because of the big inter-donor variation, which would have been decreased by a larger sample size. Additionally, harvesting a larger amount of SVF would have enabled the full comparison of all donors for all medium compositions which also would have increased the power of the analysis.

When studying the properties of the subpopulations of ASCs only one donor was used. As above, increasing the number of donors would have increased the power of the findings. However, others have characterized 10 donors with similar anthropometric characteristics and found great donor variations anyway, which despite the large sample size still enabled a general characterization of ASC subpopulations (Kalinina et al., 2015).



## CHAPTER 6. CONCLUSIONS

We found that ASCs increased keratinocyte-based wound healing, and that this was promoted by hypoxic preconditioning. Investigating the effect of hypoxic preconditioning on the proteome of ASCs, we found that proteins involved in the remodeling of ECM were significantly increased. When exploring the steps in the isolation and expansion process of ASC, it was identified that the choice of culture medium was essential. The use of hPL as culture medium supplement proved to be good alternative to FCS, especially with regards to increasing ASC proliferation. Additionally, the distribution of various subpopulations within the ASCs was affected by the choice of culture medium. When further characterizing the subpopulations of the ASCs, no difference in the stem cell characteristics was identified. However, differences in their proangiogenic potential were found. Interestingly, after a period of expansion the surface marker profile of the subpopulations was found to return back to that of the original mixed population.

### 6.1. PERSPECTIVES

To fully understand the ASC wound healing properties, appropriate wound healing models are necessary. In the absence of a comprehensive chronic wound model, effort has been made to mimic the different stages of wound healing in simplified *in vitro* models. However, the experimental systems are still not able to mimic the *in vivo* situation (Toyserkani et al., 2015). Further developing these models would be of great value of the scientific progress in this research area.

To relate the findings from the *in vitro* models to the healing of chronic cutaneous wounds in patients, it is necessary to study the actual therapeutic effect of ASCs in a clinical setting. Additionally, to know the clinical effect of hypoxic preconditioning of ASCs on the healing of chronic wounds, the method of hypoxic preconditioning should be included in the protocol for such a study.

Moreover, to further understand the effect of hypoxic preconditioning on ASCs, it would be advantageous to investigate the effect of this on the distribution of various subpopulations and the characteristics thereof. Additionally, characterizing the content of the conditioned media from the subpopulations would be interesting. This could reveal hitherto unknown aspects of distinct subpopulations.

Furthermore, to understand the predictive value of the ASC characteristics, and of the distribution of subpopulations within, it is necessary to correlate quantitative measurements of these to quantitative measurements of the clinical outcome. In this way the *in vitro* observations could be used as a biomarker to identify a good universal donor which could reduce the risk of engraftment failure and lack of clinical effect.



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